Generation mechanisms of hydrogen cyanide and ammonia in human exhaled breath

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Academic Dissertation

To be presented, with the permission of the Faculty of Science of the University of Helsinki, for public discussion in Auditorium A129, Department of Chemistry (A.I. Virtasen aukio 1, Helsinki), on 15th November, 2016 at 12 o'clock

Helsinki 2016

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ISBN 978-951-51-2678-8 (Paperback)
ISBN 978-951-51-2679-5 (PDF)
http://ethesis.helsinki.fi
Unigrafia Helsinki 2016

Abstract

Human exhaled breath contains hundreds of volatile compounds. Breath analysis is a method of seeking biomarkers among these volatiles, which could potentially serve as non-invasive medical indicators in disease diagnosis. With the development of analytical techniques more disease-related breath volatiles are being discovered. Exhaled hydrogen cyanide (HCN) and ammonia (NH_3) have been studied previously. Both of them are potential biomarkers in clinical practice. Breath HCN has been proposed as a biomarker of *Pseudomonas aeruginosa* colonization in cystic fibrosis patients, since high levels of HCN are detected in the headspace of *P. aeruginosa in vitro*. Breath NH_3 has been suggested as an indicator for monitoring hemodialysis (HD) adequacy in end-stage renal disease (ESRD) patients, since breath NH_3 is strongly correlated to blood urea. To validate breath HCN and NH_3 tests for clinical application, one has to understand the biochemical mechanisms of breath HCN and NH_3 production in the human body.

In some earlier studies, it was assumed that the oral cavity is a major production site for both breath HCN and NH_3. However, the biochemical pathways of oral HCN and NH_3 production have not been extensively studied. In this thesis, we investigated the correlation between salivary HCN and breath HCN in healthy subjects and confirmed that saliva is the main source for breath HCN. Additionally, we observed that oral anaerobes, including the genus of *Porphyromonas*, *Prevotella* and *Fusobacterium*, produce low levels of HCN *in vitro*. This implies that oral bacteria probably contribute to breath HCN generation. To explore the mechanism of breath NH_3 production, we first conducted experiments on healthy subjects and found that salivary urea is the main source of breath NH_3. We extended our study to ESRD patients during their HD treatment to investigate the connection between blood urea and breath NH_3. Through the observation of strong correlations between blood urea, salivary urea, salivary ammonia and breath NH_3, we were able to suggest a biochemical pathway for breath NH_3 production. Blood urea is diffused into saliva through salivary glands. Salivary urea is subsequently hydrolysed into ammonia by urease activity. Finally, salivary ammonia evaporates into the gas phase and becomes breath NH_3.

List of Publications

This thesis contains the following publications:

I Chen W, Metsälä M, Vaittinen O and Halonen L 2014 Hydrogen cyanide in the headspace of oral fluid and in mouth-exhaled breath *Journal of Breath Research* **8** 027108

II Chen W, Metsälä M, Vaittinen O and Halonen L 2014 The origin of mouth-exhaled ammonia *Journal of Breath Research* **8** 036003

III Chen W, Roslund K, Fogarty C L, Pussinen P J, Halonen L, Groop P-H, Metsälä M and Lehto M 2016 Detection of hydrogen cyanide from oral anaerobes by cavity ring down spectroscopy *Scientific Reports* **6** 22577

IV Chen W, Laiho S, Vaittinen O, Halonen L, Ortiz F, Forsblom C, Groop P-H, Lehto M, Metsälä M 2016 Biochemical pathways of breath ammonia (NH_3) generation in patients with end-stage renal disease undergoing hemodialysis *Journal of Breath Research* **10** 036011

The contribution of the author:

The author has prepared the manuscripts for Article I, II, III and IV. In Article I and II, the author has been responsible for all experimental work and data analysis. In Article III, she has performed most of the experimental work and been responsible for data analysis. In Article IV, she has carried out most of the experimental work and analysed all measurements.

Acknowledgements

On 16th December 2011, which was just one day after my 26th birthday, I came to the Laboratory of Physical Chemistry first time and received an introduction to laser spectroscopy from Dr. Metsälä. On 5th January 2012, when I was in China to celebrate the Chinese New Year, I received an email for the PhD position interview. On 9th February, I flew back to Helsinki and arrived at the airport at 6 am. After 7 hours at 1 pm, I was sitting in the seminar room of the Laboratory of Physical Chemistry and was interviewed by Professor Halonen, Dr. Metsälä and Dr. Vaittinen. At 4.15 pm, I received a phone call from Dr. Metsälä, who offered me a PhD position. That was the moment when my PhD journey started.

I wish to thank Professor Lauri Halonen for giving me the opportunity to pursue my PhD in his group. I want to thank my supervisor Markus Metsälä for bringing me this interesting and meaningful research. Thank you for solving all technical problems of the instruments, building up the contacts with the medical faculty, hospitals and clinics and sharing all your knowledge of the breath analysis area with me. I would also like to thank all my colleagues in the Laboratory of Physical Chemistry. Thanks for your help. Especially I want to thank Dr. Olavi Vaittinen. Thanks for your suggestions and help in experimental work.

Specially, I would like to thank (感谢), the support from my family (李秋英女士，陈瑞光先生和陈汝卿女士) and my friend Clara (Georgiana Carpov).

Contents

1 Introduction

Human exhaled breath contains hundreds of volatile compounds [1]. The major components of breath are: 74% unmodified (inhaled) nitrogen, 14-17% oxygen (reduced from 21% inhaled), 3-6% carbon dioxide, 5-6% water and 1% argon [2]. In addition to these major components, trace volatile compounds were found in breath using gas chromatography (GC) in 1960s [3]. In the early 1970s, more volatile compounds were detected in breath [4,5]. After a decade, breath volatiles started to be proposed as biomarkers in diagnosis. The potential of breath analysis serving as a non-invasive clinical tool was first comprehensively reviewed by Antony Manolis [6]. In the 1990s, the development of novel analytical techniques such as laser spectroscopy and proton-transfer reaction mass spectrometry (PTR-MS) allowed for highly selective and sensitive detection of breath volatiles [7]. Since then, even more volatiles have been discovered in exhaled breath and their potential as biomarkers in medical diagnosis has been investigated.

Due to the low concentration of breath volatiles, sample collection methods play an important role in the analysis. In general, exhaled breath consists of two major parts: alveolar air and dead-space air. The volatiles in alveolar air originate mainly from blood-borne compounds, which participate in the alveolar gas exchange. The volatiles in dead-space air originate mainly from inhaled air or are produced in the oronasal cavity and trachea. When the study of breath volatiles aims at understanding the systemic metabolic condition, dead-space air is discarded to obtain a high proportion of alveolar air during breath sampling. In addition, since CO_2 is a blood-borne compound in breath, monitoring of breath CO_2 at its constant level can indicate a collection of alveolar air [8]. When the study of breath volatiles focuses on the volatile production in the oral cavity, dead-space air is useful.

Several instrumental techniques have been used for breath analysis. Breath volatiles were first studied using gas chromatography (GC) [3]. However, most breath volatiles are present at trace levels (part-per-billion by volume or even lower). Preconcentration of breath samples is required before the analysis of volatiles by GC. In addition, the chromatographic separation stage is time consuming (up to 30-60 min). Novel instruments have been developed and validated for online and real-time measurement of the breath volatiles. These techniques include selected ion flow tube-mass spectrometry (SIFT-MS), proton-transfer reaction mass spectrometry (PTR-MS) and laser spectroscopy [9-11]. Without the steps of preconcentration

and chromatographic separation, the levels of breath volatiles can be detected in real time. The online measurement of breath using these techniques can potentially be used for bedside monitoring in the future.

There are several advantages in breath analysis when applied to clinical diagnosis. First of all, the diagnostic results can be obtained in a short time. In traditional diagnosis, the delivery of blood samples and chemical analyses required are time consuming. Using suitable instrumentation, breath can be analysed in real-time and results can be obtained immediately. . Secondly, breath analysis is a non-invasive diagnostic process. Blood tests are one of the traditional diagnostic methods. However, they are invasive and the sampling frequency is limited. Moreover, blood drawing is difficult to conduct in children, disabled or anxious patients. Compared to blood sampling, breath collection can be easily conducted, is free of stress and more convenient for patients. Furthermore, breath can be sampled more frequently than blood. Although there are many advantages in breath analysis, only few medical applications of breath analysis have been approved in recent times by the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA) for clinical practice [12]. This is partly because only little is known about the metabolic origins of the investigated breath biomarkers and because the connection between a potential biomarker and a disease is still unclear in most cases [12].

1.1 Sources of breath volatiles

Breath volatiles originate both exogenously and endogenously. A large proportion of breath volatiles are exogenous. Through environmental exposure, volatile compounds in ambient air become breath volatiles by inhalation or dermal absorption. The consumption of food and drinks also contribute to the exogenous production of breath volatiles. In addition to exogenous volatiles, there are many breath volatiles that are generated endogenously. These volatiles are the metabolites produced by the metabolic processes of organs inside the human body [2]. The metabolites diffuse into blood and circulate through the body. When the metabolites come to the surface of alveoli, they cross the alveolar membrane and enter the lungs. Eventually the metabolites become gas phase molecules, which can be detected in breath samples. Since the physiologies of patients are different from healthy subjects, the levels of specific volatile metabolites in patients can be higher or lower than in healthy subjects [13]. Thus, there is

potential in the use of breath analysis in clinical practice. Another important endogenous source for breath volatiles is the oral cavity. The human oral microbiota is complex and includes more than 300 bacterial species, together with yeasts, protozoa and mycoplasmas [14]. The metabolism of oral microbiota generates volatile products, which dissolve in saliva and further evaporate into the gas phase. Hence, the investigation of volatile compounds in saliva and bacterial headspace helps to understand the origin of those breath volatiles that are predominantly generated in the oral cavity.

1.2 Physiology of saliva and salivary volatiles

The surface of the oral cavity is bathed in two types of physiological fluid: saliva and gingival crevicular fluid [14]. Saliva is a fluid produced by three pairs of major salivary glands (parotid, sublingual and submandibular) and hundreds of minor salivary glands [15]. Saliva contains 99% water along with compounds secreted by the salivary glands. These compounds are formed in the salivary glands or diffuse from the blood vessels attached to the salivary glands. Gingival crevicular fluid is a plasma exudate. It is secreted into the gingival crevice and flows along the teeth [14]. The components of this fluid are similar to those present in blood, since gingival crevicular fluid originates from plasma. After the saliva and gingival crevicular fluids are secreted into the oral cavity, the compounds in mixed oral fluid do not only originate from blood, but are also generated by other sources, such as oral micro-organisms and food debris [15].

In my thesis, saliva samples are divided into three categories: unstimulated oral fluid, stimulated oral fluid and sublingual saliva. Unstimulated oral fluid samples are obtained by having the volunteer spit out a saliva sample directly onto a petri dish. Stimulated oral fluid samples are obtained similarly but the volunteer is first asked to chew a piece of paraffin film. This type of oral fluid contains not only saliva and gingival crevicular fluid, but also other species present in the oral cavity, such as bacteria, enzymes and food residues [15]. Stimulation by chewing a piece of paraffin is expected to mix these different components in the oral cavity. Thus, stimulated oral fluid is assumed to reflect both the systemic and oral cavity condition. Sublingual saliva samples are obtained by passive drooling, in which the saliva is drained off from the lower lip [16]. Since there is no oral movement involved in the sampling, sublingual

saliva is less contaminated by the species in the oral cavity. Sublingual saliva is the fluid excreted from the sublingual salivary gland and thus reflects mostly the systemic metabolic condition.

Volatile compounds from saliva have been investigated using GC-MS [17,18]. Al-Kateb *et al* observed 317 compounds in saliva using headspace-trap GC-MS. Among them, 268 compounds were identified [18]. The identified compounds are categorised into the following classes: aldehydes, alcohols, hydrocarbons, esters, ethers, ketones, aromatic compounds, volatile sulfuric acids, nitrogen containing compounds, acids and others [18]. Although large numbers of volatile compounds were observed in saliva, the correlation between salivary volatiles and breath volatiles has not been investigated extensively in previous studies. Hence, we set up a new methodology to investigate the properties of breath volatiles through simultaneous saliva measurement. We believe that our methodology is a powerful tool in exploring the biochemical pathways of breath volatiles.

1.3 Headspace analysis of bacterial volatiles

Bacteria produce volatile compounds in their metabolism [19-21]. These volatiles could be used to identify the bacteria both *in vitro* and *in vivo*. The *in vitro* measurement is named headspace analysis of bacterial volatiles. Bacteria are first inoculated in broth or agar for cultivation. During the growth of bacteria, volatile metabolites are generated and released into the gas phase. Carrier gas flow is commonly applied to deliver the volatiles into analytical instruments for measurement.

Due to the availability of sensitive analytical instruments, trace levels of bacterial headspace volatiles can be detected. For example, PTR-MS has been used to determine the volatiles from *Escherichia coli* and *Salmonella enterica*, in molecular mass to charge ratio range from 20 to 150 atomic mass units [21]. The mass spectra obtained from the headspace of bacterial cultures can serve as bacterial fingerprints, which can be used to identify the bacteria. Additionally, certain bacteria are capable of producing large quantities of specific volatiles. For instance, oral pathogens such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum* produce volatile sulfur compounds (VSCs) [20], which cause oral malodour. *Pseudomonas aeruginosa*, which is related to lung infections in cystic fibrosis patients, produces high levels of HCN in the

bacterial headspace [22,23]. Based on these findings, breath HCN has been proposed to be a potential biomarker in diagnosis of *Pseudomonas aeruginosa* infection.

1.4 Aims of this thesis

Exhaled hydrogen cyanide (HCN) and ammonia (NH_3) have been studied previously, due to their potential clinical applications. Breath HCN has been proposed as a biomarker of *Pseudomonas aeruginosa* colonization in cystic fibrosis patients, since cystic fibrosis patients exhibit elevated levels of breath HCN compared to healthy controls [20]. Breath NH_3 has been suggested for monitoring the progress of hemodialysis (HD) for end-stage renal disease (ESRD) patients [24-26], since the concentration of breath NH_3 is correlated to blood urea, which is an important medical indicator for the estimation of HD adequacy. To validate breath HCN and NH_3 tests for clinical practice, it is important to understand the biochemical mechanisms of breath HCN and NH_3 generation. Although previous studies have shown that the oral cavity is a dominant production site for both breath HCN and NH_3 [27], the production mechanisms in the oral cavity have not been clearly demonstrated. The aim of my thesis is to explore the biochemical mechanisms of breath HCN and NH_3 generation. A new methodology, which enables investigating HCN and NH_3 in saliva and breath simultaneously, has been developed to achieve this goal.

Article I demonstrated that the HCN levels of stimulated whole saliva are higher than those of unstimulated whole saliva. A strong correlation between salivary HCN and breath HCN was observed. These results confirm that breath HCN is generated in the oral cavity. Additionally, the concentration of breath HCN dropped after the mouth rinse with oral disinfectant. This implies that oral bacteria contribute to the generation of breath HCN. Article III showed that oral anaerobes, including the genus of *Porphyromonas*, *Prevotella* and *Fusobacterium*, were capable of producing detectable levels of HCN in the headspace of bacterial cultures. A new sampling system coupled with detection by cavity ring down spectroscopy (CRDS) was validated for the analysis of headspace HCN from both aerobic and anaerobic bacteria.

Previous studies have reported a correlation between breath NH_3 and blood urea [24,25]. We hypothesized that salivary enzymatic hydrolysis of urea was responsible for the connection between blood urea and breath NH_3. In Article II, we demonstrated that salivary urea was the

main source of salivary ammonia and breath NH_3 in healthy subjects. Further experiments were conducted on ESRD patients undergoing HD treatment. Article IV showed that blood urea was correlated strongly with salivary urea in ESRD patients and breath NH_3 concentration dropped as blood urea decreased during HD. We proved that blood urea is the main biochemical origin of breath NH_3, via enzymatic hydrolysis of urea in saliva.

2 Measurement techniques

Near-infrared cavity ring down spectroscopy (CRDS) and ultraviolet/visible (UV/VIS) liquid-phase spectroscopy were the two main techniques applied in this study. The CRDS method was used to determine HCN and NH_3 concentrations in the gas phase. UV/VIS liquid-phase spectroscopy was applied to measure urea and ammonia (NH_4^++NH_3) concentration in the liquid phase. Spearman's rank correlation test was used to analyse the correlations between the analytes in the gas and liquid phase. In this test, the p value refers to the probability of obtaining the observation results assuming the correlation coefficient r_s is zero (null hypothesis).

2.1 Gas-phase cavity ring-down spectroscopy

The CRDS method is a highly sensitive optical spectroscopic absorption technique [28]. Pulsed laser light is introduced into an optical cavity containing the gas sample. According to Beer-Lambert's law (equation 1), the absorbance (A) is proportional to the concentration of target species (c), absorption coefficient (ε) and path length (L):

$$A = \varepsilon c L. \quad (1)$$

The optical cavity is formed by a pair of high reflectivity mirrors ($R \geq 99.9\%$). The pulsed laser light reflects back and forth thousands of times between the mirrors, which enhances the effective path length of the cavity significantly. The long effective path length allows the detection of target compounds at low concentrations.

In CRDS, the concentration of target compounds is determined by measuring the time of light intensity decay. When the laser light bounces back and forth between mirrors, a fraction of photons is transmitted. The light intensity (I) of the transmitted laser is detected by a photodetector. An exponential decay of light intensity is recorded as time passes. The

exponential time constant (τ) represents the time for light intensity to decrease in value to I_0/e (equation 2), where I_0 is the light intensity entering the cavity. Hence, when the light intensity drops to I_0/e, the time is recorded and taken as the exponential time constant:

$$I = I_0 e^{(-t/\tau)}. \quad (2)$$

The exponential time constant is related to the reflectivity (R) of mirrors and losses caused by molecular absorption:

$$\tau = \frac{L}{c[(1-R)+\alpha L]}, \quad (3)$$

where c is the speed of light, L is the length of the cavity, and α is the attenuation coefficient ($\alpha = \varepsilon c$) of the sample in the cavity. The attenuation coefficient is a function of the molecular concentration in the cavity. Analytes can be identified accurately because of the unique absorption features of different molecules. The absorption of photons in the infrared region results in vibrational transitions. Because the energy difference between adjacent rotational states is usually much smaller than that of vibrational states, rotational transitions occur along with the vibrational transitions. The structure of each molecule is specific, hence the infrared energy absorbed is uniquely quantized. The relation between the energy of a photon absorbed (ΔE) and the frequency of the radiation absorbed (ν) is given by the Bohr frequency condition:

$$\Delta E = h\nu, \quad (4)$$

where h is Planck's constant. Instead of frequencies, wavenumbers ($\tilde{\nu}$) are often used in optical spectroscopy:

$$\tilde{\nu} = \frac{\nu}{c}, \quad (5)$$

where c is the speed of light. Therefore, the absorption spectrum is unique for each molecule. In the spectrum, the horizontal axis is the wavenumber scale and the vertical axis is the attenuation coefficient. In my thesis, absorption spectra are analysed by least square fitting Voigt functions to the absorption lines. The analyte concentration can be calculated from the fitted area of the respective peak. We can apply CRDS to detect gas phase HCN and NH_3 at parts-per-billion by volume (ppbv) levels.

A home-built continuous-wave CRDS instrument was used for quantification of HCN in the gas phase. The spectrometer and data analysis procedures have been described in detail before [29]. A commercial CRDS ammonia analyser (Picarro, G2103) was employed for breath NH_3 measurements in both healthy subjects and end-stage renal disease (ESRD) patients. The setup,

performance of the analyser and breath gas sampling procedures have been described in detail before [30].

2.1.1 Detection of breath HCN

To collect breath samples for HCN determination, volunteers were instructed to inhale through the nose, exhale through the mouth and fill a sampling bag with one exhalation. No attempt was made to extract the end-tidal part of the breath. The breath samples also included the dead air space contribution from the oral cavity and airways since we aimed to investigate the oral origin of breath HCN. The sampling bag was connected to inlet tubes of the CRDS instrument and the HCN concentration was determined. For experimental details, see Schmidt *et al* [29].

Breath HCN concentrations in healthy people have been studied before by SIFT-MS [31-33], CRDS [29,34] and photoacoustic spectroscopy [35]. It has been found that healthy subjects generate low levels of mouth-exhaled HCN, ranging from non-detectable to 62 ppbv [29,31,32,34,36]. The levels of mouth-exhaled HCN measured from healthy subjects in Article I were in the range of 1.8 to 9.9 ppbv, which is comparable to previous studies. They were always above the detection limit of the CRDS instrument, which is about 0.3 ppbv [29]. Thus, the measurement of breath HCN in our study was reliable and accurate.

Since only one breath sample was obtained from each volunteer, the variation between subjects might be high. It has been recommended that single breath analysis results are normalized by a physiologically based parameter, such as concentration of CO_2 in breath [37]. Normalization of the target compound has been used to reduce the sampling variation among different subjects [38,39]. Carbon dioxide originates from the lungs and thus the CO_2 level reflects the alveolar character of the breath sample. In this thesis, breath HCN and CO_2 were always determined simultaneously by CRDS (figure 1).

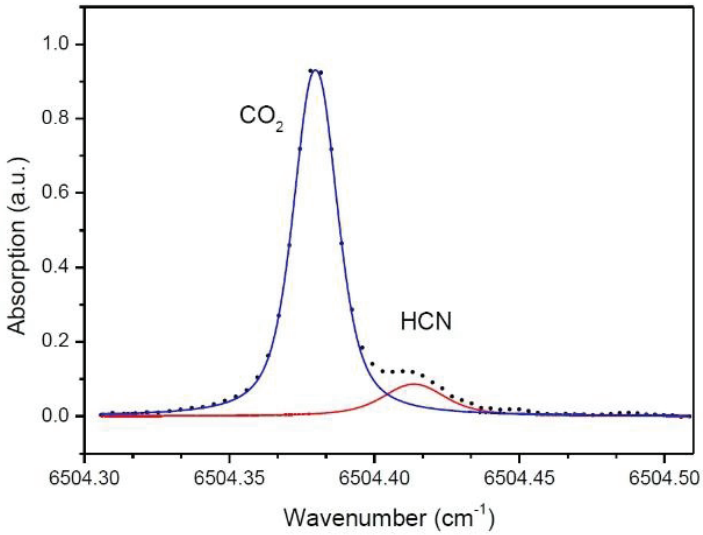

Figure 1. Representative spectrum of breath HCN measurement. The concentrations of HCN and CO_2 are 3.8 ppbv and 4.1%, respectively. Black dots are the measurement data. Red and blue lines are fits to the data using Voigt functions.

The breath HCN values presented in Article I were normalized by the breath CO_2 concentration:

$$c_{\text{norm}}(\text{HCN}) = \frac{c(\text{HCN})}{c(\text{CO}_2\%)} \times 4.1\ (\%)\ , \tag{6}$$

where the 4.1 % multiplication factor is the mean CO_2 concentration of the breath samples taken during the study.

2.1.2 Determination of salivary HCN

The headspace HCN concentration from oral fluid was used to represent the concentration of salivary HCN. Two techniques can be used to conduct headspace measurements: static and dynamic headspace sampling [40]. In static headspace sampling, the vial with a liquid sample is sealed. After waiting for certain time, the gas phase of the sample is collected and pre-concentrated using, for example, solid-phase microextraction (SPME). In dynamic headspace sampling, a flow of carrier gas is applied to deliver the target volatiles continuously into the analyser. Due to the high sensitivity of CRDS, no preconcentration step is required before the

measurement. Hence, the dynamic headspace sampling method was selected for my studies. Traditionally, the measurement for liquid headspace is conducted using a container as a carrier for the liquid sample. In our study, we used cotton swabs as saliva sample carriers (figure 2). Oral fluid samples were first collected onto a plate and then transferred to a cotton swab. The large surface area of the cotton fiber enhances the HCN evaporation from the oral fluid. In addition, to standardize the oral fluid samples obtained from volunteers, we controlled the weight of oral fluid being absorbed onto the cotton swab.

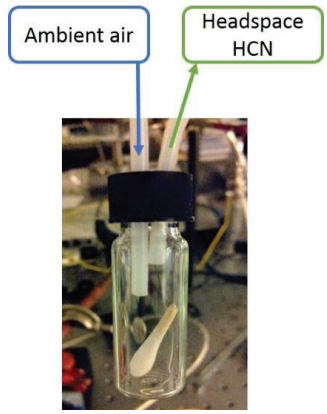

Figure 2. Dynamic headspace sampling of HCN using a cotton swab as a carrier for oral fluid.

2.1.3 Detection of HCN from the headspace of bacterial cultures

In bacterial headspace measurements, broth is commonly used as a culture medium [21,23]. In this way, the number of bacteria can be evaluated rapidly by turbidity measurements during bacterial growth. We chose solid agar as the culture medium. Since the water content in broth is high, HCN might partly dissolve into the broth. Therefore, trace levels of HCN generated by the bacteria might not be detected. For the subculture of aerobic bacteria, a single colony was selected and streaked onto an agar plate. However, anaerobic bacteria do not survive when this subculture method is used. Instead of picking up one single colony of anaerobic bacteria, we scraped all the bacterial colonies from a pre-culturing plate into phosphate-buffered saline (PBS). The PBS with bacterial suspension was utilized for further subculture. The initial colony number was larger and compared to the streaking method, the anaerobic bacteria grew better using the scraping technique.

The experimental setup for HCN measurements with aerobic bacteria is shown in figure 3. A Petri dish with the growth medium and the inoculated aerobic bacteria was placed into an airtight container and kept at 37°C. The gas tubings were heated in order to maintain the temperature at 37°C, and consequently prevent condensation of water produced by the bacteria. The headspace gas containing the volatile metabolites was transferred from the container into the measurement cell and HCN concentration was determined by CRDS.

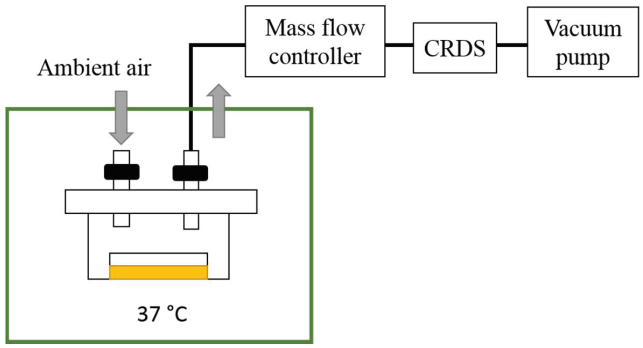

Figure 3. Experimental setup for the measurement of aerobic bacterial headspace.

For anaerobic bacteria, the sampling system set up for HCN measurements is demonstrated in figure 4. There were several differences in the setup between aerobic and anaerobic bacteria. Firstly, oxygen depleted anaerobic gas (5% CO_2, 10% H_2 and 85% N_2) was supplied to culture the anaerobic bacteria. Secondly, a pressure gauge was added into this setup to ensure atmospheric pressure, which is important for the growth of anaerobic bacteria. Thirdly, the Petri dish with growth medium was placed upside down on top of the Petri dish lid to prevent the air flow from directly flushing the agar surface, since anaerobic bacteria are sensitive to changes in the surrounding environment. This also prevented the condensate water from dripping back onto the agar surface. Furthermore, a catalyst was put in the container to deplete residual oxygen, since oxygen is detrimental to the growth of anaerobic bacteria. We observed that *P. endodontalis* did not grow without a catalyst, even though the container was filled with the oxygen depleted anaerobic gas. In addition, a water collection system was added into the setup. We noticed that the moisture level during anaerobic growth was higher than during aerobic growth. Without the water collection system, the moisture level in the container became too

high. Condensed water moved along tubings towards CRDS and eventually clogged the particle filters, which are used to protect the optical cavity.

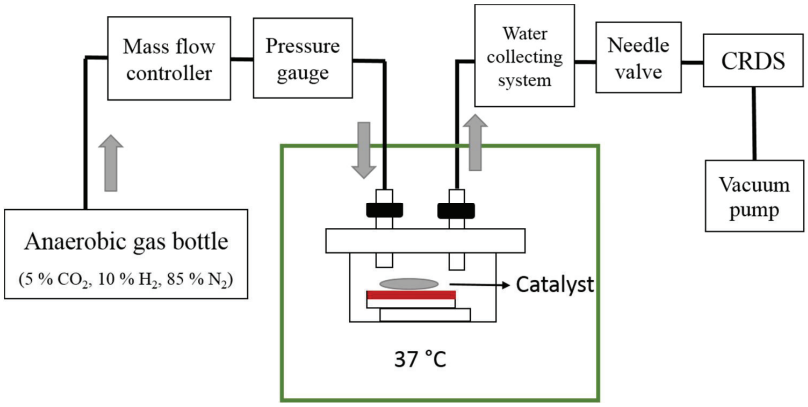

Figure 4. Experimental setup for the determination of anaerobic bacterial headspace.

2.1.4 Measurement of breath NH_3

Several techniques have been applied to measure breath NH_3, including SIFT-MS [31], ion mobility spectrometry [41] and laser spectroscopy [30,42,43]. Previous studies have shown that the levels of breath NH_3 detected in healthy subjects are between 0.07 and 2.9 ppmv [30-33,44]. The levels of breath NH_3 in ESRD patients are generally higher and range from 0.35 to 14.7 ppmv [24,25,45]. In this study, breath NH_3 was measured in real time by a commercial ammonia analyser (Picarro, G2103) based on CRDS. For healthy subjects, we measured concentrations in the range of 0.26 to 1.75 ppmv. For ESRD patients undergoing HD, we measured values in the range of 0.03 to 6.8 ppmv before, during and after dialysis. The levels of breath NH_3 detected in our studies were always higher than the detection limit 0.3 ppbv for the commercial ammonia analyser [30].

It has been mentioned that the body posture of subjects might affect the concentration of breath volatiles when the subjects are providing breath samples [2,30]. In Article II, the healthy subjects were instructed to sit straight on a chair and breathe into a fixed mouth piece. They were asked to inhale normally through the nose, exhale through the mouth, and breathe into the mouth piece, which was connected to the analyser inlet tube. The mouth-exhaled NH_3 concentration was recorded at 3 minutes of breathing, to ensure that an equilibrium of ammonia

adsorption and desorption on the surfaces of tubings and the CRDS instrument was achieved. Since ESRD patients were weak in general and needed to lay down on a bed during the HD treatment, a movable mouth piece was prepared for them to breathe. The breath sampling time for ESRD patients was only two minutes, which is one minute less than for healthy subjects. Experiments conducted on one healthy subject showed that the breath NH_3 levels measured at 2 minutes were strongly correlated (r_s=0.95, p<0.001) to the ones measured at 3 minutes (figure 5). Hence, the breath NH_3 levels obtained from patients were as reliable and accurate as the ones measured from healthy subjects. The water concentration was also measured together with the concentration of breath NH_3. In some cases, we noticed that the water level dropped to zero, although the patient's mouth was touching the mouth piece. When we communicated with these patients, they told us that they had forgotten to exhale through the mouth.

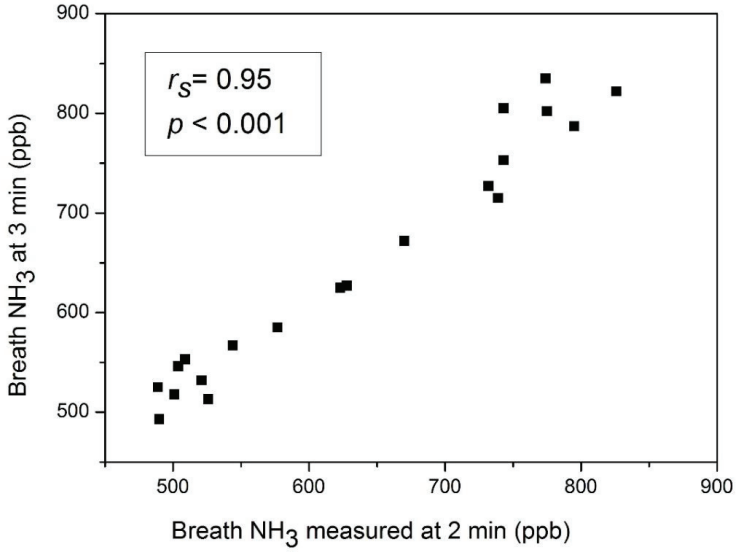

Figure 5. The levels of breath NH_3 measured at 2 min are strongly correlated with the ones measured at 3 min with one healthy subject.

2.2 UV/VIS liquid-phase spectroscopy

A UV/VIS liquid-phase spectrometer (Ocean Optics, USB4000 and USB-ISS-UV/VIS) was used to measure the concentration of urea and ammonia (NH_4^++NH_3) in the liquid phase. The broadband light from the source of the spectrometer is dispersed by a monochromator into

different wavelengths. The light intensity of the chosen wavelength decreases as it passes through the sample solution, which contains the target compounds. Transmittance (T) is defined as the ratio between transmitted light intensity (I) and incident light intensity (I_0). According to the Beer-Lambert's law, there is a logarithmic dependence between transmittance (T), absorption coefficient (ε), path length (L) and target compound concentration (c) (equation 7). Absorbance (A) depends linearly on the target compound concentration (equation 1). Thus,

$$\ln T = \ln \frac{I}{I_0} = -\varepsilon c L \qquad \text{and} \tag{7}$$

$$A = -\ln(\frac{I}{I_0}) = \varepsilon c L \ . \tag{1}$$

2.2.1 Determination of salivary urea

Urea concentrations for both stimulated oral fluid and sublingual saliva samples were determined in this study. In the following text, the salivary urea refers to the urea concentration measured from stimulated oral fluid. Urea concentration can be determined using several methods. Most of the methods belong to one of the two following categories. One is to form a derivative compound from urea, which absorbs in the UV/VIS region and, therefore, the concentration is determined by UV/VIS liquid-phase spectroscopy. The other one is to hydrolyse urea by urease into ammonium ions. The determination of ammonium ion concentration provides the levels of urea. In 1997, Knorst *et al* developed rapid analytical methods to determine urea concentration in pharmaceutical formulations [46]. The principle of these methods is that the derivatization of urea with *p*-dimethylaminobenzaldehyde forms a yellow-coloured Schiff-base [47]. The absorbance of the yellow-colored product compound is measured by liquid-phase spectroscopy and the absorbance is related to the urea concentration. This method shows good linearity, high accuracy and reproducibility, and a low limit of detection. Therefore, in our study, we used this method to determine the urea concentration.

To validate this method, we prepared 9 different concentrations of urea standard solution (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mM). We measured the absorbance at 422 nm, which is the wavelength recommended in Knorst *et al* [46]. We observed reasonably good linearity (r^2= 0.993) in the range between 0 and 5 mM of urea concentration (figure 6). Hence, this method was suitable to determine the salivary urea concentration using the Ocean Optics

spectrophotometer. It has been shown that the salivary urea levels in healthy subjects are between 1 and 7.5 mM [48]. The salivary urea levels in ESRD patients are higher, ranging from 7.5 to 42 mM [48]. The salivary urea concentration in healthy subjects ranged from 2.6 to 14.8 mM in Article II, and in ESRD patients before HD from 9.3 to 45.4 mM in Article IV. Since the salivary urea concentrations in ESRD patients were higher than in healthy subjects, the dilution factors for the salivary urea measurement in healthy subjects were lower than for ESRD patients. This ensured that the diluted salivary urea was in the range of the standard curve.

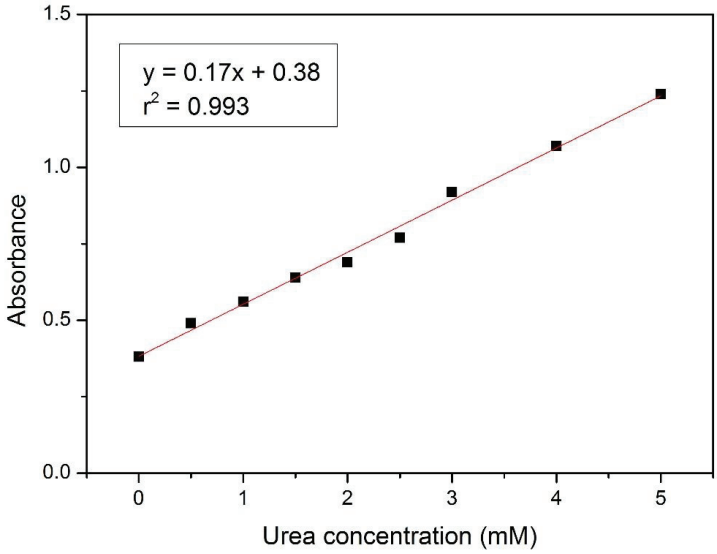

Figure 6. The standard curve of urea solutions determined at 422 nm.

2.2.2 Measurement of salivary ammonia

Salivary ammonia is present in two forms: the ammonium ion (NH_4^+) and ammonia molecule (NH_3). The average pK_a value of ammonia in blood and water is 8.95 at 37 °C [49] . According to the acid-base equilibrium, most of ammonia is present in the ammonium ion form, since the pH value of saliva is close to neutral. Only a small amount of ammonia is in the ammonia molecule form, which further evaporates into the gas phase, based on the equilibrium between the liquid and gas phase. A previous study has shown that the ammonia levels in whole saliva of healthy subjects range from 0.85 to 5.5 mM [50]. In submandibular-sublingual saliva, the ammonia levels are lower, between 0.07 and 1.0 mM [50]. Similar results were also obtained

for healthy subjects in Article II. The ammonia levels in stimulated oral fluid were between 1.3 and 9.0 mM, and in sublingual saliva they ranged from 0.065 to 0.83 mM. In the following, salivary ammonia refers to the ammonia levels in stimulated oral fluid. The level of ammonia in saliva of ESRD patients has not been studied extensively before. In Article IV, we demonstrated that levels of salivary ammonia were between 1.7 and 44 mM, which is higher than in healthy subjects.

Three methods for salivary ammonia determination were compared by Huizenga *et al* in 1982, including the indophenol, ammonium electrode and enzymatic methods [51]. In this comparative investigation, the authors concluded that the indophenol method possesses the best precision and lowest reagent cost. The indophenol method is based on chemical reactions between ammonia, phenol, sodium nitroprusside and sodium hypochlorite to form a blue coloured end product indophenol, whose concentration can be determined by measuring the absorbance using liquid-phase spectroscopy at 623 nm. Hence, through the measurement of indophenol absorbance, the concentration of ammonia is determined. We obtained a linear ammonia standard curve using the following ammonia standard solutions: 0, 25, 50, 100 and 200 µM (figure 7). The high linearity proves that the method we used to determine the ammonia concentration is valid.

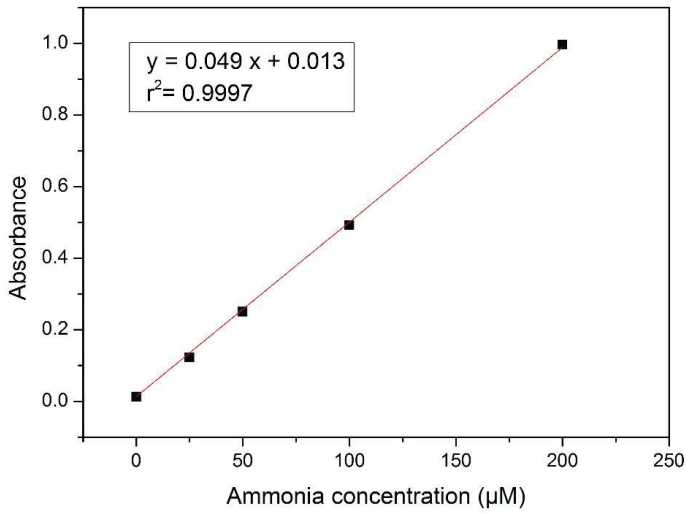

Figure 7. The standard curve of ammonia solutions determined at 623 nm.

3 The biochemical mechanism of breath HCN generation

3.1 Potential clinical applications of breath HCN

Hydrogen cyanide (HCN) is a toxic volatile compound. In nature, HCN is formed by hydrolysis of cyanogenic glycosides in seeds, roots and leaves of plants, such as bitter almonds and leaves of peach [52]. HCN is also released from bacteria through the metabolic pathway of bacterial cyanogenesis [53]. Low levels of HCN in the human body are also generated by endogenous biological processes [54,55]. Additionally, people are exposed to low levels of HCN as generated by the urban environment in industrial processes and vehicle exhausts [56]. Smokers are exposed to HCN, since HCN is abundant in cigarette smoke as a combustion product [57]. Fire victims are at risk of inhaling lethal levels of HCN, which is produced in residential fires by the incomplete combustion of nitrogen-containing polymers [58]. In fact, breath HCN has been suggested as a potential surrogate for blood cyanide test to diagnose cyanide intoxication in fire victims [59]. Breath HCN has also been proposed as a potential biomarker of *Pseudomonas aeruginosa* lung infection in cystic fibrosis (CF) patients [36], since *P. aeruginosa* produces HCN [22,60]. It has been reported that the concentration of mouth-exhaled HCN in children with *P. aeruginosa* infection was significantly higher than in those without the infection [61]. In a recent large scale study, breath HCN was shown to be a specific but insensitive marker of early *P. aeruginosa* infection in children [62]. However, in adult *P. aeruginosa* infected patients, the concentrations of mouth-exhaled HCN were not significantly elevated compared to healthy subjects [63]. Nose-exhaled HCN was recommended as a biomarker, since it is influenced less by the oral cavity [63]. However, a moderate correlation between mouth-exhaled HCN and nose-exhaled HCN has been observed, indicating that the levels of nose-exhaled HCN are also affected by the oral cavity [29]. In order to apply exhaled breath HCN in the diagnosis of *P. aeruginosa* lung infection, it is important to understand the source of breath HCN in the oral cavity.

3.2 Oral source of breath HCN

It has been assumed that the oral cavity is a production site of breath HCN [27,29,64]. We set up a new methodology to prove this assumption, by directly comparing the headspace HCN concentration from stimulated oral fluid and unstimulated sublingual saliva. We found that the headspace HCN concentration of stimulated oral fluid was higher than that of sublingual saliva. Stimulated oral fluid was assumed to reflect the oral condition, while sublingual saliva reflects mostly the systemic condition [65]. This result indicates that the HCN levels in the oral cavity were higher than systemic HCN levels of healthy volunteers. In addition, the headspace HCN from stimulated oral fluid (salivary HCN) was measured simultaneously with the breath HCN concentration. Moderate correlations between salivary HCN and breath HCN were observed both in intra- and inter-subject tests. An intra-subject test refers to the measurements conducted within a single subject. An inter-subject test refers to the experiments conducted between subjects, where data were normalized by taking individual conditions as CO_2 concentrations into account. This result implies that the main source of breath HCN for healthy volunteers is probably saliva, which is consistent with results of Lundquist *et al* [64]. As performed in my study, simultaneous measurement of the same volatiles from whole saliva and breath is a valuable new method to investigate the oral source of breath volatiles.

3.3 Bacterial source of breath HCN in the oral cavity

Oral hygiene has been assumed to affect breath HCN production [63]. Based on this, we believe that oral bacteria could be one source for breath HCN. To prove our hypothesis, we conducted oral disinfectant *in vitro* and *in vivo* tests. Corsodyl was used as the oral disinfectant to kill most of the oral bacteria and inhibit enzyme activity [66]. We recorded a drop in HCN concentration from the headspace of stimulated whole saliva after adding oral disinfectant. The remaining HCN levels were close to the HCN levels of unstimulated sublingual saliva. This result shows that the oral source of HCN is inhibited by the oral disinfectant and, therefore, only the systemic HCN levels were left. This finding was further confirmed in the oral disinfectant *in vivo* test. After a mouth rinse with oral disinfectant, both the salivary HCN and breath HCN concentrations decreased. The concentrations of salivary HCN and breath HCN stayed at low

levels for two hours after mouth rinse. This result indicates that most likely oral bacteria contribute to the generation of breath HCN.

It has been found that certain bacteria release HCN through the metabolic pathway of bacterial cyanogenesis [53]. To date, only few bacteria are known to produce HCN: *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Rhizobium leguminosarum* [53], *Burkholderia cepacia* [23,67] and *Staphylococcus aureus* [68]. Among them, *B. cepacia*, *P. aeruginosa* and *S. aureus* are found in the human body. Oral bacteria have not been studied before to investigate their HCN production properties. Hence, in this study, we first screened seven strains of oral anaerobes for HCN production. We chose oral anaerobes to investigate at the initial stage of research, because these bacteria have been found to play an important role in the production of breath volatiles [69].

Five of seven strains produced detectable amounts of HCN in 72 hours: *Porphyromonas gingivalis* ATCC 33277, *Porphyromonas endodontalis* ATCC 35406, *Prevotella intermedia* ATCC 25611, *Fusobacterium nucleatum subsp. nucleatum* ATCC 25586 and *Fusobacterium periodonticum* ATCC 33693. The highest levels of HCN from oral anaerobes were mostly detected at 48 h. The concentrations of HCN detected at this time from five strains were in the range of 1.4 to 10.9 ppbv. This is the first study to show that the genera of *Porphyromonas*, *Prevotella* and *Fusobacterium* are capable of producing HCN *in vitro*. Since *P. gingivalis* ATCC 33277 produced the highest level of HCN among the five oral anaerobes, further investigations were conducted on the species of *P. gingivalis*.

P. gingivalis is one of the better-known periodontal pathogenic micro-organisms, which contribute to the development of periodontal disease. Previous studies have shown that *P. gingivalis* produces volatile metabolites, such as volatile sulphur compounds (VSCs) [70]. However, none of the previous studies have demonstrated that *P. gingivalis* produces HCN. Since we found in the screening test that *P. gingivalis* produced the highest level of HCN among the tested anaerobes, we selected three reference strains (ATCC 33277, W50 and OMG 434) and one clinical isolate (4753E) of *P. gingivalis* to further investigate their HCN production properties.

The HCN in bacterial headspace was measured at 24, 48 and 72 hours. We defined these measurements as offline measurements. From the experiments, we found that ATCC 33277, W50 and 4753E produced similar concentrations of HCN, while OMG 434 produced much

lower concentrations. To further investigate the quantitative change in HCN production during the growth of *P. gingivalis*, we determined the dynamic profile of HCN production by an online measurement, in which the HCN concentration was determined every 20 minutes for 72 hours. We found that *P. gingivalis* ATCC 33277 produced the highest amount of HCN, while *P. gingivalis* OMG 434 produced the least, which was consistent with the observation from the offline measurements. These findings indicate that both offline and online measurements in our study are valid and reliable.

Traditionally, the growth curve of bacteria is determined simultaneously to show the effect of bacterial metabolic activity on the production of volatile metabolites. However, in our study it was impossible to determine the real-time growth curve for *P. gingivalis*, because the culturing was done on agar instead of broth. From broth culturing, the optical density (OD) can be determined in real-time to estimate the bacterial number. It has been previously demonstrated that CO_2 is one of the metabolites in the metabolic pathway of *P. gingivalis* [19]. Hence, CO_2 measured simultaneously in the experiment could serve as an indicator for metabolic activity of *P. gingivalis*. We observed an increase in the HCN levels as the CO_2 levels went up in the selected strains of *P. gingivalis*. This indicates that the metabolic activity of *P. gingivalis* has an effect on its HCN production capacity, and HCN is probably one of the volatile metabolites of *P. gingivalis*. In addition, colony-forming units (CFU) were determined at the end of HCN measurements at 72 hours. The quantity CFU is used to estimate the number of living bacteria. The CFUs were in the range of $(0.7 – 3.9) \times 10^{10}$, which showed that the samples of *P. gingivalis* were always in good growing conditions. This further confirms that our sampling setup is valid for measuring headspace HCN from anaerobic bacteria.

Although the HCN levels produced by the oral anaerobes (less than 10 ppbv) were much lower than by aerobic *P. aeruginosa* (hundreds to thousands of ppbv), one should note that these studies were conducted *in vitro*. The HCN production by oral anaerobes *in vivo* is still unexplored and the growing conditions in the oral cavity are different from the agar plate. The co-cultures of oral anaerobes might also affect the levels of HCN production. To further demonstrate the contributions of oral anaerobes in the production of breath HCN *in vivo*, we suggest to measure the level of breath HCN and bacterial number of oral anaerobes simultaneously and investigate their correlation. The oral anaerobes investigated in this study

are well-known periodontal pathogens. Determination of breath HCN could be a potential tool for the detection of pathogenic oral anaerobes in the future.

4 Biochemical pathways of breath NH_3

4.1 Ammonia metabolism in the human body

Ammonia in the human body originates mainly from the metabolism of diet protein (figure 8). After food intake, protein in the stomach and intestines is initially broken down into amino acids, ammonia and other nitrogenous compounds [43]. Ammonia diffuses from the digestive system into the blood. Ammonia in blood is present mostly in the ammonium ion (NH_4^+) form, and only a small fraction is in the ammonia molecule (NH_3) form. In the following text, we use ammonia as a general term to represent both forms (NH_4^++NH_3), except for further notification. Part of ammonia in blood passively diffuses to salivary and sweat glands. It can be detected in both saliva and sweat [71-73]. Another part of blood ammonia is directed to the liver and converted to urea, a less toxic nitrogenous compound. After ammonia transfers from blood into the liver, it first combines with CO_2 to form carbamoyl phosphate. This is the first step for ammonia in the urea cycle. After several chemical reactions, carbmoyl phosphate is eventually transformed to urea. The normal blood ammonia concentration in healthy subjects is 11–50 μmol/L [74]. In hepatic disease patients, the blood ammonia levels increase to abnormally high levels, because the urea cycle is impaired. When urea is formed, it is released into the blood again, absorbed by the kidneys and filtered out of the body by urine. However, if a subject has renal disease, urea cannot be removed from the blood by kidneys. This leads to high blood urea concentrations in these patients.

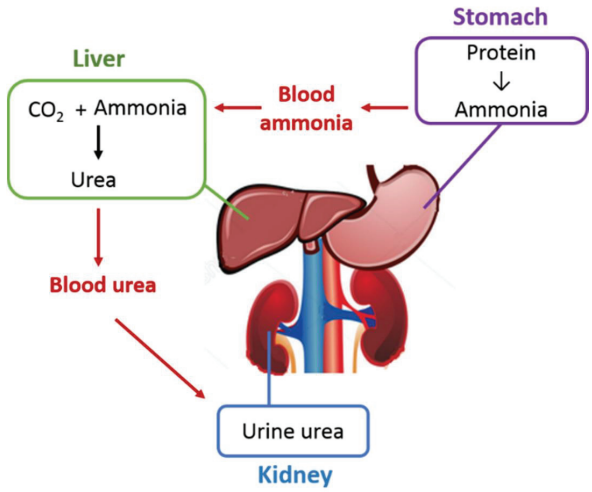

Figure 8. The urea cycle inside the stomach, liver and kidneys.

4.2 Potential clinical application of breath NH_3 in ESRD patients

Breath NH_3 has been proposed as a non-invasive biomarker in several different clinical applications, including the diagnosis of *Helicobacter pylori* infection [75] and the assessment of halitosis [76]. It has also been found that breath NH_3 is statistically significantly correlated to blood urea in end-stage renal disease (ESRD) patients undergoing hemodialysis (HD) [24,25,41,45,77], implying that breath NH_3 is a potential indicator in the monitoring of HD treatment.

The kidneys of ESRD patients fail to remove waste products sufficiently, leading to the accumulation of toxic compounds in the body. One of the renal replacement therapies is to use an HD machine. A semipermeable membrane functions as a dialyzer in the HD machine, which is used for the removal of waste products from blood. The HD adequacy is normally determined by *Kt/V*, which is calculated according to the Daugirdas equation [78]. The quantity *K* stands for the dialyzer clearance, indicating the blood flowrate through the dialyzer, and *t* is the duration of HD treatment. Thus, the product *Kt* represents the total volume of fluid, in which urea is cleared. The quantity *V* is the total volume of body water. A value of *Kt/V* above 1.2 is normally indicative of a successful HD treatment. Blood urea is measured to estimate the HD adequacy, because urea clearance was found to be correlated with the clearance of other toxins during hemodialysis [79]. The HD adequacy is determined once every month, by measuring the

levels of blood urea to obtain *Kt/V*. Due to the infrequent monitoring, patients might undergo insufficient HD for a long time, go unnoticed and have their health deteriorate irreversibly [80]. Therefore, it is essential to set up a methodology to monitor the HD adequacy frequently.

Uremic fetor, which is described as a urine-like and ammoniacal odour, is commonly found in the breath of ESRD patients [81]. It has been shown that breath NH_3 declined in ESRD patients during HD and there was a moderate correlation between exhaled NH_3 and blood urea nitrogen [24,25,41,45,82,83]. However, Endre *et al* demonstrated oscillatory patterns in breath NH_3 of certain patients [45]. To apply breath NH_3 test to monitor HD adequacy, one should understand the biochemical pathways of exhaled NH_3 generation and how breath NH_3 reflects the pathophysiology of ESRD patients undergoing HD.

4.3 Hypotheses of breath NH_3 generation mechanism

In previous studies, two different mechanisms were put forward to explain the decline of exhaled NH_3 in ESRD patients during HD (figure 9). In the first hypothesis, the decline of breath NH_3 is directly due to the drop of blood ammonia (NH_4^++NH_3) during HD. It was assumed that there is an equilibrium between blood urea and blood ammonia, and therefore, exhaled NH_3 reflects blood ammonia levels via gas exchange in the lungs [77]. When the level of blood urea is high, some urea is metabolized back to ammonia, leading to a high blood ammonia concentration [83]. In the process of HD, both blood urea and ammonia are removed [84]. Since blood ammonia was claimed to be correlated to breath NH_3 [85], the level of breath NH_3 was assumed to decrease along with blood ammonia. However, it was found that the levels of blood ammonia were within normal range in chronic kidney disease (CKD) patients and there was no significant difference between pre- and post-dialysis blood ammonia levels [86]. Correlation between blood ammonia and breath NH_3 was not observed in recent studies [87,88].

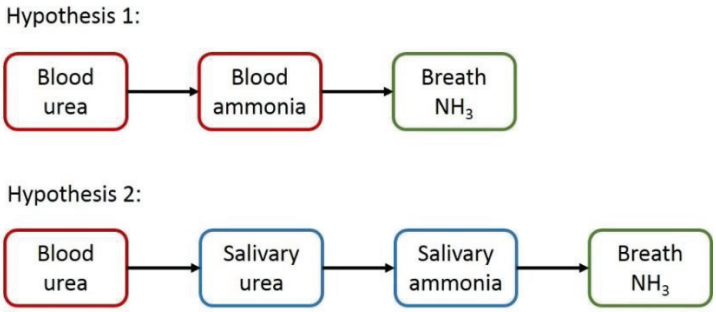

Figure 9. Two hypotheses of breath NH_3 generation mechanism.

In the second hypothesis, the reduction of blood urea in HD leads to a decline of salivary urea which is connected with exhaled NH_3 via enzymatic hydrolysis by urease. Since urea diffuses from blood to saliva through the salivary glands [72,73], a strong correlation between blood urea and salivary urea has been observed in several studies [48,89-91]. Salivary urea is further hydrolysed by oral bacterial urease into ammonia [92,93], which becomes a major source of breath NH_3. The mechanism of second hypothesis was demonstrated in healthy subjects in Article II by our new methodology, which explored the origins of breath volatiles by analysing their relevant counterparts in saliva. We observed that there was no significant difference in the urea concentration between stimulated oral fluid and sublingual saliva. This indicates that the urea in the oral cavity is produced mostly by systemic processes. A significant difference in the ammonia concentration was found between stimulated oral fluid and sublingual saliva. This result implies that ammonia in the oral cavity is predominantly generated inside the mouth and the systemic contribution is limited. We further demonstrated that there were strong correlations between salivary urea, salivary ammonia and breath NH_3 in both intra- and inter-subject tests. This confirms that ammonia in the oral cavity originates from salivary urea. Based on our findings in healthy subjects, we hypothesized that the same mechanisms apply to ESRD patients.

4.4 Mechanism of breath NH_3 generation in ESRD patients

We measured the levels of blood urea, salivary urea, salivary ammonia (NH_4^++NH_3), salivary pH and breath NH_3 from 12 ESRD patients before, during and 30 min after the HD treatment.

Through the analysis of respective correlations, we demonstrated a biochemical pathway for breath NH_3 in ESRD patients undergoing HD.

The levels of blood urea, salivary urea, salivary ammonia (NH_4^++NH_3) and breath NH_3 decreased gradually in most patients during the dialysis treatment. A strong correlation (r_s=0.77, p<0.001) between blood urea and salivary urea was observed in ESRD patients undergoing HD. This confirms that the level of salivary urea reflects the level of blood urea (figure 10, step A). Salivary ammonia declined as salivary urea dropped. Strong correlations between salivary urea and ammonia (NH_4^++NH_3) were observed in most of the patients. This indicates that salivary urea is a major source for ammonia production (figure 10, step B). We noticed that the correlations between salivary urea and ammonia in intra-subject tests were stronger than in inter-subject test. This is probably due to the variations of urease activity in different patients. We also noticed that the correlations between salivary ammonia and breath NH_3 in intra-subject test were much stronger than in inter-subject test. This arises because the salivary pH value varies between patients (figure 10, step C). To minimize the pH variations between patients, we calculated the salivary NH_3 concentration using the levels of the salivary ammonia (NH_4^++NH_3) and the pH value according to the Henderson-Hasselbalch equation [94]. A strong correlation between salivary NH_3 and breath NH_3 (r_s=0.85, p<0.001) was observed in inter-subject test (figure 10, step D). This result indicates that the salivary pH value plays an important role in the levels of breath NH_3. Finally, a moderate inter-subject correlation between blood urea and breath NH_3 (r_s=0.60, p<0.001) was observed in our study (figure 10, step E).

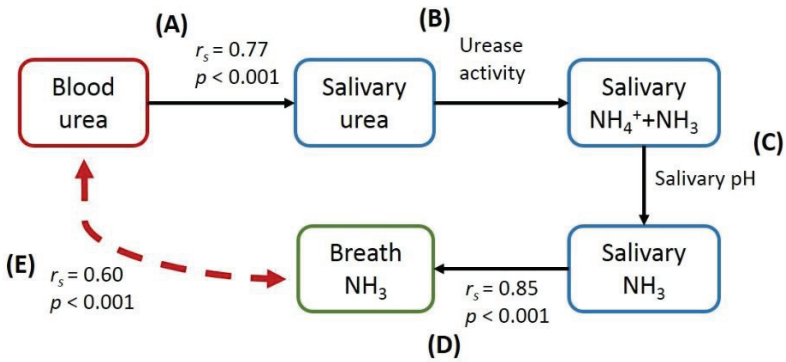

Figure 10. Biochemical pathways of breath NH_3 generation in ESRD patients undergoing HD treatment.

Salivary ammonia levels declined along with salivary urea in most patients. However, there were two anomalous cases (P8 and P6), in which the salivary ammonia levels did not follow the change in salivary urea (figure 11). A significant drop of salivary ammonia and pH was observed in P8 during food intake at around 50 min. We have demonstrated that a high proportion of salivary ammonia is generated in the oral cavity. Hence, we assume that the salivary ammonia was washed out during eating and drinking. After a while, the level of salivary ammonia started to increase again. However, the urea concentration in saliva dropped continuously due to the removal of blood urea by HD treatment. The change in oral condition probably destroyed the connection between salivary urea and ammonia. For P6 no statistically significant correlation between salivary urea and ammonia was found. The changes in salivary ammonia levels were seemingly random. Similar results were also observed by Endre *et al*, who demonstrated an oscillatory pattern in breath NH_3 levels of certain ESRD patients during HD [45]. There are several possible reasons for this. Firstly, the patient may not have chewed properly the paraffin pellets which were used to obtain stimulated saliva samples. This could cause a large variation in the measured salivary ammonia levels. Secondly, the use of oral disinfectant or certain medications might affect the oral urease activity. Thirdly, it has been reported in addition to urease, arginine deiminase also generates ammonia in the oral cavity [92]. Arginine might contribute in a higher proportion to ammonia production in this patient.

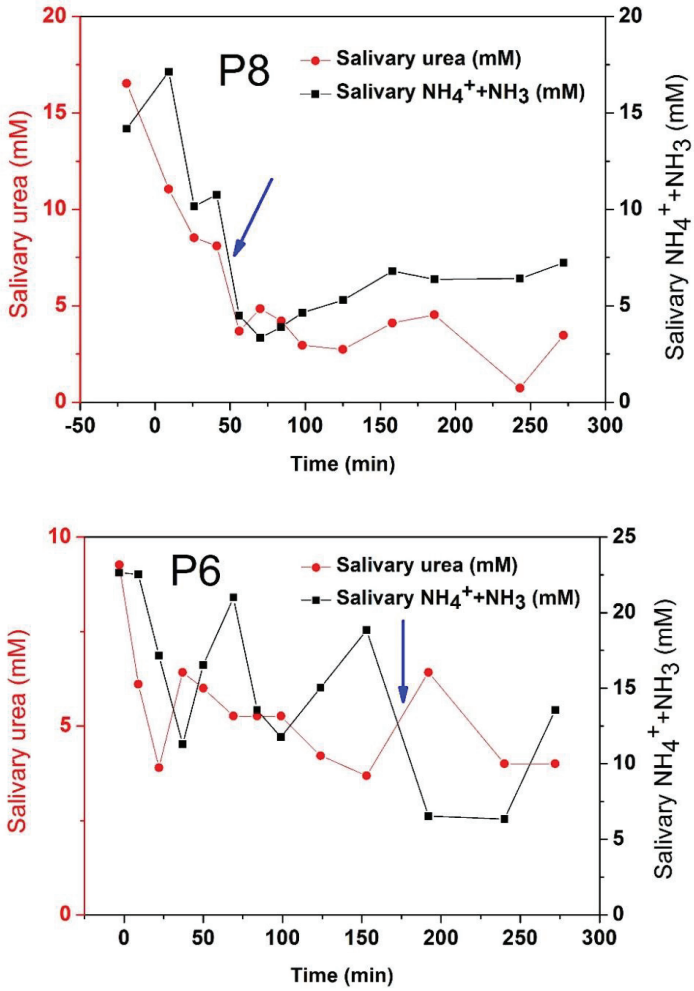

Figure 11. Anomalous patterns in salivary urea and ammonia (NH_4^++NH_3) observed for P8 and P6. Arrows indicate time of food intake.

5 Conclusion

Breath analysis is a potential diagnostic tool of the future. Compared to the standard blood test, breath analysis has several advantages. First of all, the method is non-invasive and produces little discomfort to the patient. Secondly, body produces breath continuously. Breath can be sampled as frequently as needed, allowing continuous monitoring of the patient. Sample collection is also straightforward and some analytical techniques can provide real-time analysis

results. The application of this diagnostic tool requires more research not only on instrumental development, but also in understanding the origin and metabolic pathways of the biomarkers. Sensitive analytical instruments, based on e.g. PTR-MS, SIFT-MS and laser spectroscopy, allow the detection of breath trace gas constituents down to pptv levels. However, without a detailed understanding of the sources of breath volatiles in the body, the diagnostic value of the breath tests remains debatable. Potential clinical applications have been proposed for both breath HCN and NH_3. In this thesis, I have studied the sources and production mechanisms for these volatile breath species. I hope that my work will help to facilitate the application of these volatile biomarkers in clinical practice.

A new methodology was set up for the investigation of the origin and biochemical pathways of HCN and NH_3. The main idea of this new methodology is to investigate the connection between salivary volatiles and breath volatiles. Firstly, through the concentration comparison of the target compounds between stimulated oral fluid and unstimulated sublingual saliva, the oral origin or systemic origin could be determined. If the levels of target compounds are lower in unstimulated sublingual saliva than in stimulated oral fluid, we conclude that the target compounds are probably of oral origin. Secondly, the simultaneous measurement of target compounds in stimulated saliva and mouth-exhaled breath helps in elucidating the mechanism of breath volatile production in the oral cavity. In saliva, we can analyze not only the volatile species themselves but also non-volatile sources that eventually produce the breath volatiles through, for example, enzymatically catalyzed reactions taking place in the oral cavity. In addition, I have set up a novel headspace sampling system, in which both aerobes and anaerobes were grown and HCN from the headspace of bacterial cultures was detected. Previous studies have mainly focused on the HCN headspace analysis of aerobic bacteria, such as *Pseudomonas aeruginosa* and *Burkholderia cepacia*. HCN production in anaerobic bacteria has not been investigated before. This new setup could be widely applied to the screening of *in vitro* HCN production by both aerobic and anaerobic bacteria. It can help find more cyanogenic bacteria in the human body and understand the bacterial contribution to breath HCN production. It is essential to understand the origin of breath HCN before breath HCN is approved as a valid biomarker in clinic practice in the future. Furthermore, this is the first study to show that oral pathogens are capable of producing HCN. Although the levels of HCN produced *in vitro* by

oral pathogens are much lower than by *P. aeruginosa*, I believe that *in vivo* studies are required to clarify the HCN production ability of these bacterial species inside the oral cavity.

In this thesis, my hypothesis on the generation mechanism of breath NH_3 has been demonstrated. I successfully showed that urea in blood diffuses into saliva, which becomes a main source for salivary ammonia. The value of salivary pH determines the amount of ammonia molecule (NH_3) evaporating into the gas phase. It is beneficial to confirm the generation mechanism of breath NH_3. This not only helps to explain the correlation between blood urea and breath NH_3, but also unravels the confounding factors which will influence the potential clinical applications of breath NH_3. Although the biochemical pathways and influencing factors of breath NH_3 are known, more clinical research is required to validate the breath NH_3 measurement on estimation of HD adequacy in ESRD patients, including standardization of measurement protocols. One of the important remaining questions that should be studied in the future is the effect of urease activity on breath NH_3 levels. I have shown a correlation between salivary urea and ammonia but a measurement of urease activity influencing the salivary ammonia levels is still lacking.

In conclusion, breath analysis is a promising non-invasive tool in disease detection and therapeutic monitoring in the future. Unravelling the biochemical pathways is an essential step for breath volatiles to become valid biomarkers in clinical practice.

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